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Phorbol Myristate Acetate and Catechol as Skin Cocarcinogens in SENCAR Mice

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The enhancement of the carcinogenicity of benzo(a)pyrene (B[a]P) and β-propiolactone (BPL) by the mouse skin cocarcinogens phorbol myristate acetate (PMA) and catechol were examined in female SENCAR mice, 30 per group. The carcinogen and cocarcinogen were applied simultaneously, three times weekly for 490–560 days. B(a)P and BPL were used at constant doses of 5 and 50 μg, respectively, in all experiments. PMA was used at three doses, 2.5, 1.0, and 0.5 μg per application, and catechol was used at one dose, 2 mg per application. Control groups included animals that received carcinogen only, cocarcinogen only, acetone only, and no treatment. The carcinogenicity of B(a)P and BPL were enhanced by the cocarcinogens, particularly in terms of tumor multiplicity. For both carcinogens, the most marked cocarcinogenic effects were observed at the lowest dose of PMA used (0.5 μg per application). This observation applied for days to first tumor, animals with tumors, tumor multiplicity, and incidence of malignant skin tumors. Catechol applied alone did not induce any tumors; with PMA alone there were significant incidences of benign and malignant tumors, e.g., at a dose of only 0.5 μg per application, 15 of 30 animals had 28 tumors, 5 of which were squamous carcinomas. In two-stage carcinogenesis experiments with 7,12-dimethylbenz(a)anthracene (DMBA) as initiator and PMA as promoter, SENCAR mice showed a greater susceptibility to tumor induction when compared to ICR/Ha mice used in earlier work. This susceptibility was most notable in terms of rate of tumor appearance and tumor multiplicity.

Introduction

The two-stage model for chemical carcinogenesis was first described in the classic work of Berenblum (*1,2*), who used mouse skin as the route of exposure. At the same time, Berenblum showed that remarkable enhancement of the carcinogenic activity of benzo(a)pyrene [B(a)P] occurs when croton oil or its concentrate, croton resin, and B(a)P are applied simultaneously and repeatedly to mouse skin. The latter type of experimental protocol was referred to by Berenblum as cocarcinogenesis (*1*). Since that time, the mouse skin test system has been used in many laboratories for studies on two-stage carcinogenesis and, to a lesser extent, cocarcinogenesis, and these endeavors have been reviewed (*3,4*). The two protocols as used by Berenblum are summarized in Table 1.

One result of the early work was the development of mouse lines that would be particularly sensitive to tumor induction in two-stage carcinogenesis. The development of sensitive mice was first accomplished in the pioneering work of Boutwell (*5*), who explored the development of skin tumor-susceptible (STS) mice by careful inbreeding experiments using two-stage carcinogenesis for selection of STS mice. The necessary requirements of inbreeding and the small sizes of colonies used for the development of STS mice resulted in the decline of reproductive performance in these mice. This poor breeding performance was alleviated by crossbreeding male STS mice with female Charles River CD-1 mice. The details and history of the susceptible mice thus developed were recently described (*6*). These mice, originally maintained at the Oak Ridge National Laboratory, have since than been named SENCAR mice.

Table 1. Protocols for two-stage carcinogenesis and cocarcinogenesis.

	Protocol
Two-stage carcinogenesis	Single application of initiator (DMBA, B(a)P, or BPL) followed by repeated applications of promoter (PMA), i.e., sequential application of chemicals.
Cocarcinogenesis	Repeated and simultaneous application of carcinogen (B(a)P or BPL; low dose) and cocarcinogen (PMA or catechol).

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In the course of work on carcinogens, tumor promoters, and cocarcinogens in tobacco smoke condensate, it became necessary to examine some major components of tobacco smoke for their tumor promoting and/or cocarcinogenic properties. In human exposure to cigarette smoke, there is simultaneous and repeated intake of aromatic hydrocarbon carcinogens and many other chemicals, some of which were considered potential cocarcinogens. Many of the tobacco phenols, which were suspected as tumor promoters based on the finding that phenol itself is a weak tumor promoter (*7*), proved to be inactive as tumor promoters using the two-stage protocol (*8*). This proved to be the case for such compounds as catechol, pyrene, fluoranthene, undecane, and several others in a series of structurally unrelated chemicals that were neither active as tumor promoters in two-stage carcinogenesis nor carcinogens (*8,9*).

For continued studies on environmentally important factors, particularly chlorinated humic acids, which occur in drinking water, it became relevant to explore the use of SENCAR mice because of their reported increased susceptibility to skin tumor induction (*6*).

In the chemical and biological work that resulted in the isolation and structure elucidation of the phorbol esters from croton oil, random-bred ICR/Ha mice were used (*10*). These mice were also used in our subsequent work on the promoter phorbol myristate acetate [PMA, also referred to as (12-O-tetradecanoylphorbol-13-acetate TPA)], including dose-response studies, the effect of aging in two-stage carcinogenesis studies, and other studies (*4*). The same mouse strain was also used in our earlier studies on mouse skin cocarcinogens (*9*). Hence, it became relevant to compare our earlier results using ICR/Ha mice with results of similar studies using SENCAR mice.

When the work described in the present report was undertaken in early 1980, reports indicated that SENCAR mice are more susceptible to mouse skin tumor induction than CD-1 mice (*6*) and BALB/c mice (*11*) in two-stage carcinogenesis using several endpoints of bioassays. In subsequent work, DBA/2 mice were found to be as sensitive as SENCAR mice, also in two-stage carcinogenesis studies using PMA as a promoter (*12*). The sensitivity of various mouse strains, including SENCAR mice, to complete carcinogenesis and to the initiation-promotion protocol has been rank ordered (*13*).

The work described in this report deals with the use of SENCAR mice in skin tumor induction mainly using cocarcinogenesis protocols with B(a)P and β-propiolactone (BPL) as carcinogens together with the mouse skin cocarcinogens PMA and catechol. The findings obtained with SENCAR mice, described here are compared with the earlier studies using ICR/Ha mice (*4,9,14*).

Methods

Animals, Housing, and Treatment

Female SENCAR mice were obtained from Oak Ridge National Laboratories (Oak Ridge, TN) at approximately 4 weeks of age and were put on test at approximately 6 weeks of age. There were 30 animals per group. All chronic experiments were preceded by short-term evaluations lasting 4 to 6 weeks to determine acceptable doses for chronic exposure. The doses selected for short-term evaluation were based on those used earlier with the same chemicals in ICR/Ha mice (*9,15*). For the two-stage experiments, the test duration was 550 days and for cocarcinogenesis, the test duration was 490 or 560 days. The dorsal skins of all mice were shaved at the beginning of experiments and then as needed during the tests. Animal housing and care was the same as that used before (*9*).

All animals were weighed at bimonthly intervals, and records were maintained. Animals were observed for tumor appearance daily; tumors were scored and observations were charted once per month. All solutions were applied to the dorsal skin in 0.1 mL acetone by micropipette except for 7-12-dimethylbenz-(a)anthracene (DMBA), which was administered by subcutaneous injection in the left flank in 0.05 mL tricaprylin per injection. The appropriate vehicle and no-treatment control groups were included; treatment groups and doses of chemicals used are presented under Results. In two-stage carcinogenesis experiments, a single dose of DMBA was followed two weeks later by thrice-weekly application of PMA. In the cocarcinogenesis experiments, the two agents (B[a]P or BPL and cocarcinogens PMA or catechol) were applied simultaneously for the duration of the test. Animals were killed at the conclusion of tests or when in moribund condition and necropsied. Skin lesions were excised, sectioned, and submitted for histopathologic diagnosis. Lesions were counted as papillomas when they reached a size of 1 mm^3 and persisted for more than 30 days. Lesions that regressed by day 30 or earlier were not counted in the total cumulative tumors.

Chemicals

DMBA (Eastman Organic Chemicals, Rochester, NY) was purified by recrystallization from 95% ethyl alcohol to constant melting point of 122.5–123°C; fresh solutions were prepared immediately before use in bioassays. B(a)P (Aldrich Chemical Company) was recrystallized from benzene:methanol (1:3) to constant melting point of 174–176°C. B(a)P solutions were prepared once per month from freshly recrystallized material. BPL (Sigma Chemical Company, St. Louis, MO) was tested as >99% pure by gas chromatography and used as such. Solutions of BPL were prepared freshly once per week. Catechol (Fisher Scientific Co., Fairlawn, NJ) was purified by recrystallization from toluene, mp 100–103°C. Its purity was checked by thin-layer chromatography on silica gel (chloroform:ethyl acetate, 1:1, R_F 0.68). All solutions were prepared in anhydrous acetone, and all pure chemicals and their solutions were stored in amber-colored containers at 4°C. PMA used in the two-stage experiments was prepared from croton oil, purified, and its purity checked

Table 2. Two-stage carcinogenesis in SENCAR mice.[a]

Treatment and dose, μg	Time to first tumor, days	Number of mice with tumors/ total tumors	Number of mice with malignant tumors	
			Squamous carcinoma	Sarcoma
Subcutaneous[b]				
DMBA, 0.5, PMA 0.5	93	14/22	5	1
DMBA, 0.1, PMA, 0.5	120	16/32	10	0
DMBA, 0.5, acetone, 0.1 mL	540	1/1	1	0
DMBA, 0.1, acetone, 0.1 mL	—	0	0	0
Topical[c]				
DMBA, 5, PMA, 0.5[d]	44	30/692	23	2
DMBA, 5, acetone, 0.1 mL	140	4/8	3	0
PMA, 0.5	170	13/22	4	1
Acetone, 0.1 mL	374	2/2	0	0
No treatment[e]	513	1/1	0	0

[a] Initiation by subcutaneous injection and by topical application of DMBA; PMA by topical application; Test duration 550 days; Median survival time > 550 days; 30 female mice per group.

[b] Injected subcutaneously in 0.05 mL tricaprylin in left flank once only, followed 14 days later by three times weekly application of PMA in 0.1 mL acetone to dorsal skin.

[c] Single dorsal skin application of DMBA in 0.1 mL acetone, followed 14 days later by three times weekly application of PMA in 0.1 mL acetone.

[d] Test duration: 400 days, median survival time: 311 days.

[e] Sixty mice.

by thin-layer chromatography as described before (*15*). PMA for the cocarcinogenesis experiments was obtained from Lifesystems Co. (Newton, MA) and from CCR Inc. (Eden Prairie, MN). Its purity was checked by thin-layer chromatography as before (*15*). Solutions for cocarcinogenesis bioassays were prepared by combining proper concentrations of carcinogens and cocarcinogens once weekly so that the final concentrations in the bioassay test solutions were those shown under "Results." They were stored at 4°C in amber-colored containers. Solutions of BPL combined with catechol were stored at −20°C. To ensure that no reaction occurred between BPL and catechol, bioassay solutions stored under these conditions were checked for purity by gas chromatography six days after preparation. Only the peaks for the pure components were noticed, and traces of possible reaction products or other impurities were not detectable.

Results

The sensitivity of SENCAR mice to two-stage carcinogenesis on skin using protocols that were used in earlier work (*4,14,15*), was examined with DMBA as an initiator at two doses (0.5 μg and 0.1 μg) by the subcutaneous route and at 5 μg by the topical route. PMA was applied topically as a promoter at 0.5 μg/application throughout. The low dose of PMA used, 0.5 μg per application, was based on short-term evaluations of SENCAR mice for skin irritancy. The results of this experiment are shown in Table 2. Topical application of DMBA followed by PMA gave the expected high incidence of both benign and malignant tumors in the DMBA–PMA group and also showed that DMBA and PMA by themselves induced benign and malignant tumors. The latter tumor incidences were markedly higher than those observed earlier (*4,14,15*) in comparable control groups with ICR/Ha mice.

DMBA administered subcutaneously at 1/10 and 1/50 of the dose used for skin initiation was still effective as an initiator, but the tumor incidences were markedly lower than those induced by skin application.

In the second series of experiments, the carcinogens B(a)P or BPL were applied simultaneously and repeatedly with the mouse skin cocarcinogens PMA or catechol. In these experiments, PMA was used at three dose levels with a constant dose of carcinogen. Catechol was used at one dose only. The results of these experiments together with the results from appropriate control groups are shown in Table 3. Using B(a)P or BPL as carcinogens, higher incidences of tumors were induced with decreasing doses of PMA, from 2.5 to 0.5 μg/application. Days to first tumors ranged from 49 to 85 days. The rates of tumor appearance reflected clearly the inverse relationship between dose of PMA and total numbers of tumors as shown in Figures 1 and 2. In the control group, B(a)P or BPL alone resulted in late appearance of first tumors and lower incidences of tumors after 500 days on test than were seen with simultaneous application of PMA or catechol.

Discussion

In the evaluation of the relative susceptibility of two or more strains of mice to skin tumor induction by chemicals, it is necessary to consider: (1) treatment regimen, which can be a single chemical applied once only or repeatedly (carcinogenesis), single treatment with an initiator followed by repeated application of a promoter (two-stage carcinogenesis) or repeated treatment with two chemicals applied simultaneously (cocarcinogenesis); (2) dose-response information; (3) class of chemical used, e.g., direct-acting or indirect-acting carcinogen, type of promoter, or type of cocarcinogen; (4) duration of treatment and subsequent observation; (5) animal survival; (6) incidence of spontaneous tumors; and (7) experimental observations that include: time to first tumor, rate of tumor appearance, tumor multiplicity, tumor regression, and incidence of malignant tumors. Because of the differences in goals of various investigators, it is rarely possible to obtain all the desired information.

In the present work, the main purpose was to examine the susceptibility of SENCAR mice to tumor induction in two-stage and cocarcinogenesis experiments and to compare these findings with the same chemicals used in our earlier work in the same experiments with ICR/Ha mice. Only one two-stage carcinogenesis experiment comparable to an earlier experiment (*15*) was carried out in this work, the results of which are shown in Table 2. In both experiments, the dose regimen was one application of DMBA, 5 μg, followed by PMA, 0.5 μg, thrice weekly for the duration of the tests. In ICR/

Table 3. Cocarcinogenesis in SENCAR mice, 30 female mice per group.

Carcinogen + cocarcinogen and dose, μg/application[a]	Test duration, days	Median survival time, days	Time to first tumor, days	Mice with tumors/ total tumors	Mice with malignant tumors: Squamous carcinoma	Mice with malignant tumors: Sarcoma
Treatment groups						
BP, 5 + PMA, 2.5[b]	560	372	50	27/334	21	1
BP, 5 + PMA, 1.0	490	344	49	27/423	16	0
BP, 5 + PMA, 0.5	490	322	49	29/473	22	0
BP, 5 + Catechol, 2000[b]	560	310	179	30/192	25	0
BPL, 50 + PMA, 2.5[b]	560	447	69	16/36	10	1
BPL, 50 + PMA, 1.0	490	437	85	24/99	11	2
BPL, 50 + PMA, 0.5	490	404	57	28/124	19	0
BPL, 50 + Catechol, 2000[b]	560	>560	153	14/43	9	0
Control groups						
BP, 5[b]	560	555	295	24/79	19	0
BPL, 50[b]	560	>560	293	2/4	1	0
PMA, 2.5[b]	560	515	277	10/12	4	0
PMA, 1.0	490	>490	141	15/24	8	0
PMA, 0.5	490	>490	85	15/28	5	0
Catechol, 2000[b]	560	>560	—	0	0	0
Acetone, 0.1 mL	500	>500	—	0	0	0
No Rx (65 mice)	520	>520	—	0	0	0
No Rx (60 mice)	570	>570	—	0	0	0

[a] Carcinogens: B(a)P and BPL; cocarcinogens: PMA and catechol applied to the dorsal skin simultaneously in 0.1 mL acetone three times weekly for the duration of the test except where noted. For preparation of solutions, see "Methods."

[b] Treated three times weekly for 120 days, then two times weekly for 440 days due to severe irritancy of PMA at dose used.

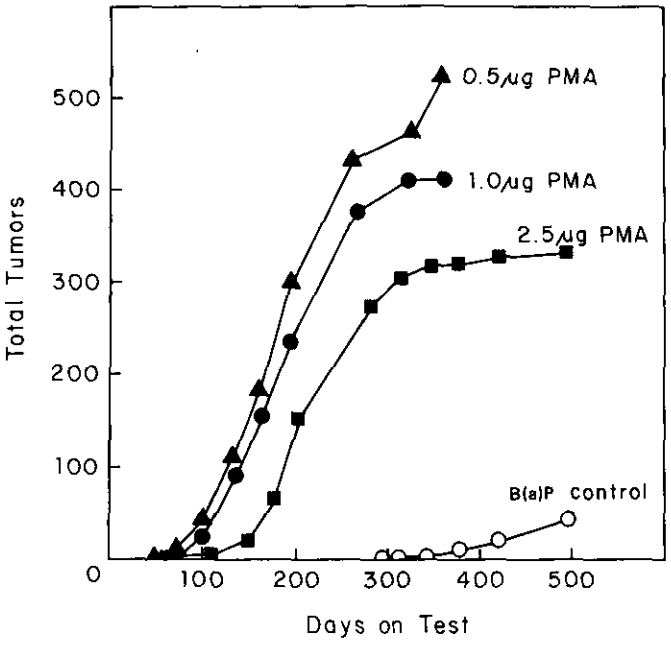

FIGURE 1. Rate of tumor appearance in skin cocarcinogenesis with 30 female SENCAR mice per group: B(a)P at 5.0 μg, PMA at doses indicated. Applied simultaneously three times weekly.

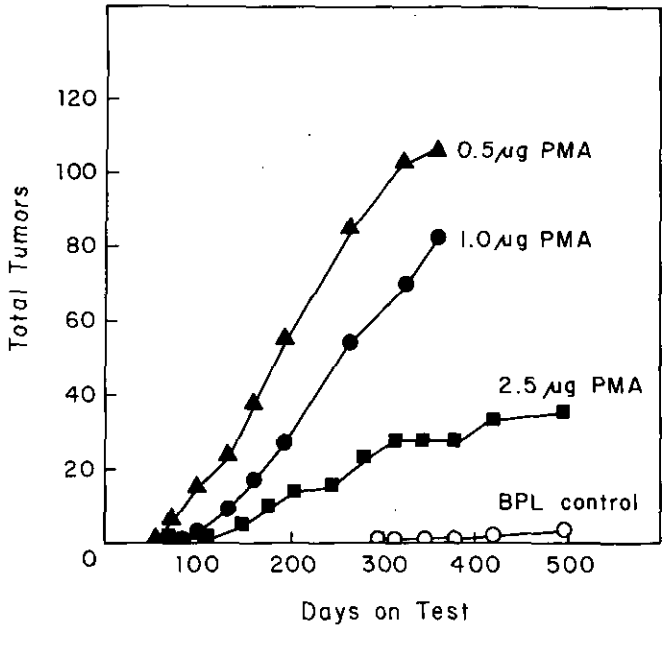

FIGURE 2. Rate of tumor appearance in skin cocarcinogenesis with 30 female SENCAR mice per group: BPL at 50 μg, PMA at doses indicated. Applied simultaneously three times weekly.

Ha mice, 13 of 20 animals had tumors after 365 days on test, of which 3 had squamous carcinoma of the skin (*15*). In SENCAR mice, all 30 animals had tumors, of which 23 had squamous carcinomas and 2 had sarcomas. The test duration was 365 days for ICR/Ha and 400 days for SENCAR mice, hence the tests were comparable. There was little difference in days to first tumors, which was 36 days for ICR/Ha mice and 44 days for SENCAR mice. The most marked difference between these mice was in tumor multiplicity, which averaged 2.7 per mouse for ICR/Ha mice and 23.6 for SENCAR mice. In addition, the rate of tumor appearance was

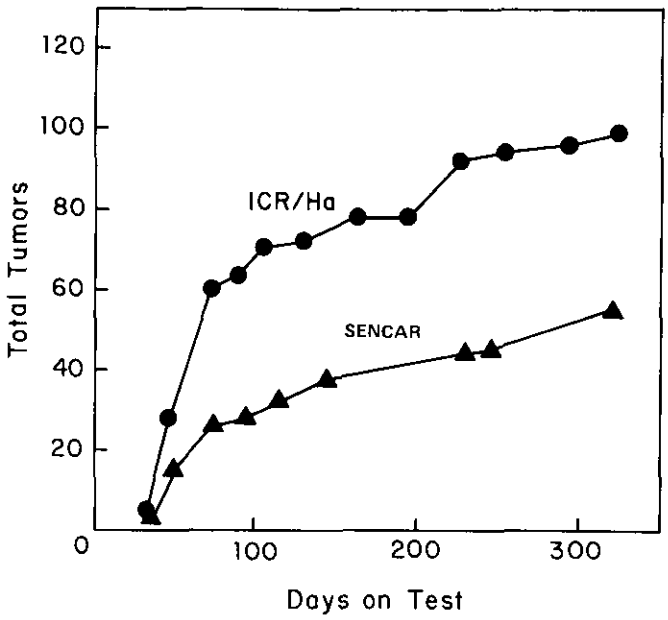

FIGURE 3. Rate of tumor appearance in two-stage carcinogenesis with ICR/Ha and SENCAR mice, 20 females per group; 30 mg BPL in 0.1 mL acetone once only, followed 3 days later by 5 μg PMA in 0.1 mL acetone, 3 times weekly for 320 days.

much more rapid in SENCAR mice compared to ICR/Ha mice, which is similar to what has been observed in comparing SENCAR mice to other mouse strains (*13*).

The differences in tumor response between the two strains depend on the dose of PMA used as promoter. SENCAR mice are more sensitive to the irritant effect of PMA than ICR/Ha mice. The optimal dose of PMA, in terms of mice with tumors, tumor multiplicity, and rate of tumor appearance, for SENCAR mice was 0.5 μg per application (Table 2), whereas for ICR/Ha mice, it was 25 μg (*15*). This comparison is based on a dose of 5 μg of DMBA as initiator, a 14-day interval between initiation and promotion, and thrice weekly application of PMA. It is not possible to extrapolate from this type of experiment to other tumor initiators or promoters or to mouse skin assays for complete carcinogens.

In another comparative two-stage carcinogenesis experiment, BPL was used as the initiator, 30 mg, once only in 0.1 mL acetone administered topically, followed by PMA, 5 μg per application, three times weekly for 320 days using a 3-day interval between initiation and promotion (*14*). There were 20 female ICR/Ha and SENCAR mice per group. The rate of tumor appearance in this experiment is shown in Figure 3. Unlike many other comparative studies that showed SENCAR mice to be the more sensitive strain (*13*), ICR/Ha mice had more rapid tumor appearance and final tumor yield. Days to first tumor were close to 35 days for both groups. The final tumor yields were 16 SENCAR mice with 55 tumors (three carcinomas) and 18 ICR/Ha mice with 100 tumors (two carcinomas, one sarcoma).

The two differences between this experiment and the one shown in Table 2 are the use of an indirect-acting carcinogen (DMBA) in one case and a direct-acting carcinogen (BPL) in the other; and the use of a higher dose (5 μg) of PMA with BPL initiation compared to 0.5 μg with DMBA initiation. It is likely that the high dose of PMA resulted in decreased tumor yields in SENCAR mice, as was observed in the cocarcinogenesis experiments summarized in Table 3.

It has been observed in studies with PMA as promoter in two-stage carcinogenesis experiments that control groups receiving PMA alone develop a low incidence of benign and malignant skin tumors (*16*). In the present study using only SENCAR mice, the induction of benign and malignant skin tumors with PMA alone at three dose levels, shown in Table 3, was considerably higher than that observed with PMA in ICR/Ha mice (*15*). The lowest dose used, 0.5 μg PMA thrice weekly, induced 28 tumors in 15 animals, 5 of which had carcinomas (Table 3). Similar findings were made when the effect on SENCAR mice of PMA alone was compared with the effect of PMA on other mouse strains, CD-1 mice (*6*) and BALB/c mice (*11*), both of which were found to have lower tumor incidences than SENCAR mice.

The importance of dose-response studies is illustrated in Figures 1 and 2, in which the results of B(a)P or BPL applied simultaneously with PMA (cocarcinogenesis) at three dose levels are compared. In both cases, the highest rate of tumor appearance and highest tumor yields were obtained at the lowest dose of PMA used, 0.5 μg. These results are probably due to increased skin inflammation and hyperplasia as the dose of PMA is increased. During the course of the tests listed in Table 3, the skin-damaging effects of 2.5 μg of PMA applied thrice weekly became apparent. These effects were remedied by decreasing the frequency of application to twice weekly, as noted in Table 3.

In the present work, studies on mouse skin cocarcinogenesis were extended by examining the phenomenon in SENCAR mice, by including in the present study the direct-acting carcinogen BPL, and by including the use of several doses of PMA as a cocarcinogen. The results of the last set of experiments emphasized the importance of using several doses of PMA in these cocarcinogenesis experiments, as was done in earlier studies on dose-response in two-stage carcinogenesis with PMA as promoter (*15*). The potency of PMA as a skin irritant necessitates careful consideration of this factor in comparing different mouse strains in skin carcinogenesis studies using the two protocols given in Table 1.

In addition to PMA, the cocarcinogen catechol was used in the present work. The high dose of catechol used in the study was arrived at on the basis of early short-term evaluations of its skin irritancy (*9*). Since it did not show any notable skin irritancy at 2 mg per application, this dose was selected for use in cocarcinogenesis experiments. No dose-response experiments

Table 4. Conclusions: skin tumorigenesis in female ICR/Ha and SENCAR mice.

Ranking	Response
SENCAR > ICR/Ha	Tumor multiplicity
	Earlier peak response
	Both 1 and 2 occur with polycyclic aromatic hydrocarbons and PMA
SENCAR = ICR/Ha	Minimum latent period
	Pattern of morphologic development of skin tumors by two-stage and cocarcinogenesis protocols
	Incidence of squamous carcinoma of the skin in lifetime tests
ICR/Ha > SENCAR	Tumor induction in two-stage carcinogenesis after BPL initiation and promotion by PMA

were carried out with this chemical, which is remarkably simple in chemical structure, yet biologically active as cocarcinogen. Catechol is completely devoid of tumor-promoting or carcinogenic activity in mouse skin using both ICR/Ha (*9*) and SENCAR mice (present study). Further work with this compound is indicated, both in terms of biological activity, particularly dose response, and mode of action.

From the work described in this report and findings reported earlier in mouse skin cocarcinogenesis experiments from the same laboratory with ICR/Ha mice (*9,14*), limited comparative conclusions, listed in Table 4, were made. These conclusions apply only to the experiments performed in our laboratory, within the dose ranges used, for the duration of treatment, and for the chemicals used. Extrapolation to other experiments and experimental conditions as enumerated at the beginning of this Discussion was not attempted.

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